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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A23J 1/14, A23L 1/211, 1/015, A23J 3/34 — A1

(11) International Publication Number: WO 96/39859

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/GB96/01345

(22) International Filing Date: 7 June 1996 (07.06.96)

(30) Priority Data:
PCT/GB95/01324 7 June 1995 (07.06.95) EP
(34) Countries for which the regional or international application was filed: GB et al.
9524353.1 29 November 1995 (29.11.95) GB

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS OF TREATING PLANT MATERIALS WITH HYDROLYTIC ENZYMES

(57) Abstract

The invention relates to methods for treating plant material such as sunflower seed meal with water and a hydrolytic enzyme in an aqueous environment to increase its nutritional value and to enzymatic methods for converting plant derived material into useful products.

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METHODS OF TREATING PLANT MATERIALS WITH HYDROLYTIC ENZYMES

The invention relates to methods for making useful products from precursors such as plant materials. The invention also relates to methods for converting said products into still further useful derivatives.

Large amounts of plant material are produced as a by-product of various manufacturing processes in the form of a pulp. For example, sunflower seed meal is produced as a by-product of sunflower oil manufacture. Sunflower seed meal has a high protein content (approximately 40%) which comprises significant amounts of nutritionally important amino acids. Thus it has potential as a human food or an animal feed. Until now, such plant material has received very little attention as a resource. Some plant material is used for animal feed, but its nutritional value is lowered by the presence of endogenous phenolic compounds, principally chlorogenic acid, an ester of caffeic acid and quinic acid. The phenolic compounds reduce the nutritional value of the plant material by binding to proteins, especially to essential amino acids such as lysine. Phenolic compounds are also known to cause discolouration of feed materials and extracted proteins [Smith and Johnsen, *Cereal Chem.*, 1948, 25, 339]. Removal of these phenolics will give a more acceptable foodstuff material. Attempts have been made to remove the phenolic compounds by solvent extraction, but in general these have failed principally because chlorogenic acid is relatively insoluble in organic solvents, and aqueous extraction procedures tend to remove both the chlorogenic acid and the nutritious protein from the plant material. (See Tranchino *et al*, Qual. Plant Plant Foods Hum. Nutr., 1983, 32, 305 for further information.) Extraction into organic solvents makes the process logistically inconvenient because of the necessity for sequential extractions (see Sripad *et al.*, J. Biosci., 1982, 4, 145). Clearly, it would be desirable for the plant material to be

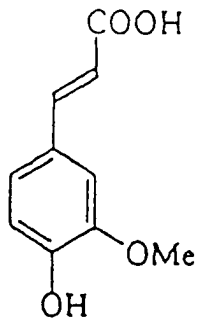
put to better use.

According to the invention there is provided a method of treating plant material to increase its nutritional value comprising exposing it to a hydrolytic enzyme in an aqueous environment and removing phenolic compounds produced by hydrolysis using solvent extraction. Some suitable solvents are listed in Table 2.

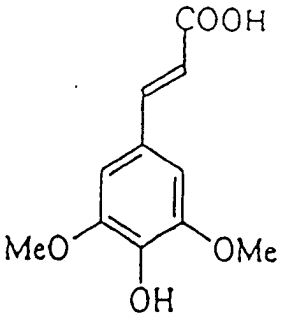
The term *plant material* as used herein includes material such as the meal or pulp produced by mechanical processing of plants. The plant material typically comprises the residue or meal produced by mechanical processing of plant material or the residues remaining after the extraction of seed oils eg. sunflower seeds, rape seed, olives, potatoes, cereal grains, such as wheat or corn, coffee, soya bean, tobacco, grapes, sugar beet and such like.

By removing products of hydrolysis, such as caffeic acid produced by hydrolysis of chlorogenic acid as described, for example, in Example 1, the nutritional value of the remaining plant material is increased considerably. In the case of plant material in the form of processed rape seed, the method of the invention can be used to remove the excessively bitter taste caused by sinapine, an ester of sinapic acid and choline.

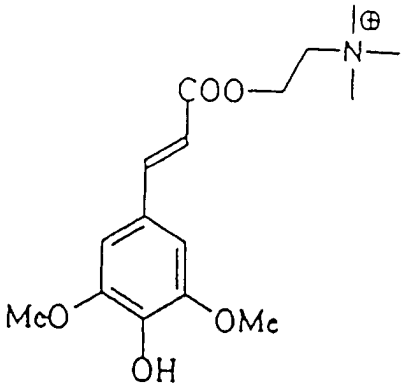
Thus the phrase "increase the nutritional value" used herein embraces improving the palatability of the plant material and, by removing discolouring phenolic compounds, the suitability/acceptability of the treated plant material as a food for humans or animals.



Ferulic acid



Sinapic acid



Sinapine

Whilst the methods of the invention can be used to improve the nutritional value of plant and plant-derived materials as food, the extracted products of hydrolysis such as caffeic acid, quinic acid, sinapic acid, choline, coumaric acid and ferulic acid are also valuable.

In a further aspect the invention provides a method of preparing a phenolic compound comprising treating a plant material by exposing it to a hydrolytic enzyme in an aqueous environment and removing the phenolic compound.

Preferably, the phenolic products are removed by solvent extraction.

Preferred hydrolytic enzymes for use in the methods of the invention are those which exhibit activity on an ester bond, hydrolysing the carbon oxygen bond to produce acid and alcohol moieties. These are the enzymes classified in the Enzyme Classification recommendations as E.C. 3.1. and subgroups thereof.

Preferably the hydrolytic enzyme does not exhibit a substantial proteolytic activity as such activity may reduce the nutritional value of the protein

component of the treated plant material. Conveniently, the enzyme is isolated from *Humicola, Bacillus* and *Aspergillus* species or is isolated from porcine liver. Suitable enzymes include those listed in Table 1.

Preferred enzymes for use in the methods of the invention can be isolated by the selection procedure detailed in Example 10. This procedure involves screening candidate enzyme preparations for chlorogenic acid hydrolysis activity and the absence of a substantial proteolytic activity against bovine serum albumin. It will be appreciated that similar selection procedures can be used for other phenolic compounds of interest and other proteins could be employed in the optional proteolytic activity selection step.

The above screening methods form further aspects of the invention.

The enzyme preparation marketed by the Enzyme Process Division of Novo Nordisk, Denmark under the trademark Celluzyme® is particularly preferred for use in the methods of the invention. Celluzyme® is a cellulolytic enzyme preparation produced by submerged fermentation of the fungus *Humicola insolens*. The enzyme complex is used in the laundering of cotton fabrics or mixed fabrics containing cotton.

Celluzyme is listed on relevant inventories of chemicals which are approved for use in cosmetics and foodstuffs, eg. EINECS (European Inventory of Existing Chemical Substances) and TSCA (Toxic Substances Control Act). Celluzyme is classified in Chemical Abstract Service as "Cellulase" (CAS No. 9012-54-8). The corresponding Enzyme Classification number (International Union of Biochemistry) is E.C. 3.2.1.4.

Another Novo Nordisk enzyme preparation useful in the methods of the invention is Bio-Feed™ Plus, a carbohydrase preparation produced by submerged fermentation of *Humicola insolens*. The enzyme hydrolyzes arabano-xylans and β-glucans into oligosaccharides and some mono-, di- and trisaccharides. Bio-Feed Plus contains other carbohydrase activities, including cellobiase, hemi-cellulase and cellulase.

A further enzyme preparation useful in the methods of the invention is sold by Biocatalysts Limited of Pontypridd, Wales, under the tradename Pectinase 162. Pectinase 162 is a wide activity spectrum pectinase derived from the *Aspergillus* species. The pectinase is effective on both soluble and insoluble pectins.

Advantageously, the plant material is derived from sunflower, rape, cereals including wheat, corn, potato and rice, or combinations thereof. However, skilled persons will appreciate that a variety of material from other plants can be used in the methods of the invention including material from tomatoes, olives, sugar beet or other available plants.

Preferably the method of the invention further comprises precipitating and isolating protein from the treated plant material.

Preferably the methods of the invention further comprise the step of administering the treated plant material or protein to a human or animal.

In a second aspect, invention provides a food comprising treated plant material or protein obtainable by the method of the invention. The food may be for human consumption or may be in the form of an animal feed.

The invention also relates to the use of the treated plant material or protein

in a method of making a food product for human or animal consumption.

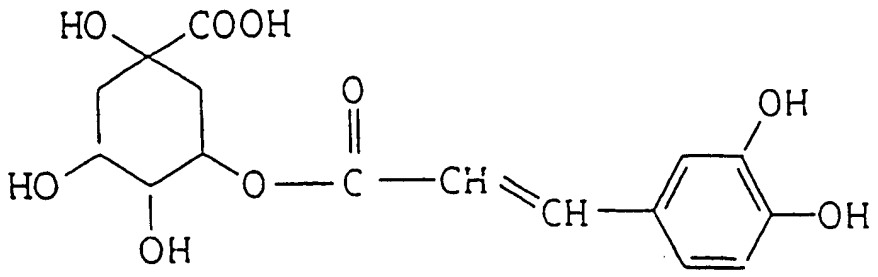
In a third aspect, the invention provides a method of converting sinapine to sinapic acid and choline comprising hydrolysing the sinapine enzymatically.

In a fourth aspect the invention provides a method of making sinapic acid and choline comprising the method of the third aspect and the further step of removing the products of hydrolysis.

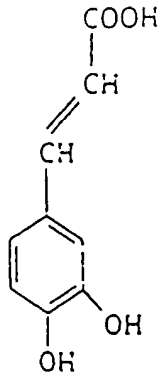
In a fifth aspect the invention provides a method of producing ferulic acid comprising treating plant material by exposing it to a hydrolytic enzyme in an aqueous environment and removing the ferulic acid produced. Although plant material containing ferulic acid precursors is widespread throughout the plant kingdom, the plant is preferably wheat, or rice.

In a sixth aspect the invention provides a method of making caffeic acid and/or quinic acid by treating plant material containing chlorogenic acid by exposing it to a hydrolytic enzyme in an aqueous environment and removing, for example by solvent extraction, the products of hydrolysis.

Chlorogenic acid is a commonly available compound of the formula:



Caffeic acid is a molecule of the formula:



This molecule is known *per se* but is only of use as a research chemical. It is a natural molecule found in plants such as coffee. It is extremely difficult and expensive to produce and is available only in very small quantities. At present, the method of production of caffeic acid is by solvent extraction from plant tissue and column chromatography.

The present invention provides a novel inexpensive method for producing caffeic acid and derivatives thereof.

An advantage of the methods of the invention is that they do not require large liquid volumes and they allow direct enzyme treatment of the plant material. Preferably, the ratio of plant material to water is from 1:10 or less dilute (ie. is ($\geq$ 1):10) and more preferably from 1:5 to 1:2, especially 1:3. Smaller liquid volumes are advantageous because a smaller total volume of solvent has to be extracted following the reaction to remove the products of hydrolysis. Furthermore, less water has to be removed in drying the residual treated plant material. Thus, the methods of the invention can be carried out on thick slurries of plant material containing a minimum amount of water so that the total volume of material that has to be processed is reduced to a minimum.

The protein component of the treated plant material can be extracted by adjusting the pH of the plant material solution to an alkaline pH so that the protein is solubilized. The remaining solid material is removed, for example by filtration, and the protein is precipitated from the solution by reducing the pH to an acidic pH. The precipitated protein can be collected, for example by filtration, and dried. Suitable methods are described by Niazi, A.H.K. *et al*, Sci. Int. (Lahore), 1994, **6**, 249-250 and Trachino, L. *et al*, Qual. Plant Plant Foods Hum Nutr, 1983, **32**, 305-334. The protein produced can be used in a variety of applications such as food for humans or as animal feed.

The methods of the invention allow waste plant material to be converted into products which have considerable commercial value.

Table 1: Enzymes capable of hydrolysing chlorogenic acid

Enzymes	Source	Source organism
Biofeed Plus	N	*Humicola insolens*
Biofeed Beta	N	*Bacillus subtilis Humicola insolens*
Fungamyl Super 5000MG	N	-
Celluzyme	N	*Humicola insolens*
Energex	N	*Aspergillus niger*
Fungamyl L	N	*Aspergillus oryzae*
Viscozyme L	N	*Aspergillus niger*
Macer 8(O)	B	*Aspergillus sp.*
Macer 8(W)	B	*Aspergillus sp.*
Macer 8(R)	B	*Aspergillus sp.*
Macer 8(FJ)	B	*Aspergillus sp.*
Pectinase 162	B	*Aspergillus sp.*
Depol 40	B	-
Depol 165	B	-
Esterase(E3128)	S	Porcine liver
Protease (P4755)	S	*Aspergillus oryzae*
Pectolase	G	*Aspergillus niger*

Key:
N = Novo Nordisk
B = Biocatalysts Ltd
S = Sigma
G = Grinstead

Table 2

List of solvents suitable for extraction of caffeic acid from plant material such as treated sunflower meal

Solvent(s)	Vol(ml)	Mass of meal	Mass of extract	Amount of caffeic acid in extract (%)
Ethyl acetate	300	50	0.52	21.1
Isopropyl alcohol (IPA)	300	50	8.73	1.89
IPA/Water (1:1)	300	50	9.5	2.23
IPA/Water (4:1)	300	50	11.52	1.08
n-Butanol	300	50	1.73	13.1
Ethanol	300	50	9.29	1.5
Hexanol	300	50	1.04	4.13
Chloroform/methanol /water (25:25:1)	100	5	0.94	4

Preferred embodiments of the invention will now be described by way of example with reference to the following examples.

Example 1

Extraction and hydrolysis of chlorogenic acid in sunflower meal to caffeic acid by Celluzyme

Pelleted sunflower meal (50g) was added to an aqueous solution (175ml) containing Celluzyme (2g; Novo Nordisk). The reaction was mixed manually and heated via water bath (45°C). The pH of the reaction mixture was monitored using a pH meter and held at pH 7.0 by the addition of aqueous sodium hydroxide (1.0 M). The hydrolysis of chlorogenic acid was monitored by High Performance Liquid Chromatography (HPLC) analysis and when completed after 5 hours incubation the reaction was terminated by addition of concentrated hydrochloric acid (0.7 ml). The reaction mixture was extracted with ethyl acetate (200 ml + 100 ml) which was separated, pooled, dried over sodium sulphate and dried with heating *in vacuo*.

Yield of extract = 0.52 g (caffeic acid content 21%)

Yield of treated sunflower meal (oven dried) = 40.84g.

HPLC assay conditions for chlorogenic acid and caffeic acid

Samples for assay were directly injected onto a C_{18} reverse phase chromatography column on a Waters high pressure liquid chromatography rig. The carrier phase was a 20:80 v/v mixture of acetonitrile:water containing 1% v/v acetic acid eluting at 2 ml/min. The substrate and products of the reaction were detected by a UV detector monitoring at 320nm.

Example 2

Effect of sunflower meal to water ratio on hydrolysis of chlorogenic acid from sunflower meal by Celluzyme

Data for the effect of sunflower meal (SFM) to water ratio is summarised in the following table. This demonstrates that the overall yield of caffeic acid from the meal is comparable for the 1:10 and 1:3 ratios although the latter resulted in a larger mass of extract. The lower ratio is advantageous in that the reaction has a smaller volume per mass of meal, a smaller volume has to be extracted to remove the caffeic acid and less water has to be removed in drying the meal.

Ratio (SFM:water)	Yield of extract from SFM (%)	Caffeic acid in extract (%)	Caffeic acid yield from SFM (%)
1:10	2.2	46	1.12
1:5	2.2	34	0.74
1:3	3.2	37	1.18

Example 3

Effect of pH and temperature on activity of Celluzyme towards chlorogenic acid producing caffeic acid

Rate of hydrolysis of chlorogenic acid demonstrated that the optimum pH of Celluzyme was pH8. Optimum temperature is in the range 45-55°C; at 65°C the enzyme was found to be unstable. pH7 was used for hydrolysis reactions as the rate of oxidation of chlorogenic acid and caffeic acid was lower than at pH8.

Rate of chlorogenic acid hydrolysis: pH 4 < 5 < 6 < 7 < 8 > 9
Temp. 55°C > 45°C

Example 4

Enzymatic production of ferulic acid from wheat germ

Method

Wheat germ (10g) was added to water (100ml, pH7.0) in a water jacketed reaction cell (45°C) and stirred using a magnetic follower. A pH stat was used to hold the reaction at pH7.0 by dosing aqueous sodium hydroxide (1.0M). The amount of ferulic acid extracted in one hour was determined by HPLC and Celluzyme (200 mg; Novo Nordisk) added. After 8000 sec. the amount of ferulic acid extracted was again measured by HPLC: Area of peak = 1.11×10^8 (~33 μg/ml), retention time (RT) = 5.13 min.

An identical control reaction without addition of Celluzyme was performed. After 8000 sec. the amount of ferulic acid extracted was again measured by HPLC: Area of peak = 3.31×10^7 (~2 μg/ml), RT = 5.06 min.

HPLC conditions: C_{18} reverse phase column, 2ml/min flow rate, 290 nm detector absorbance, acetonitrile/water (20:80) + 1% acetic acid mobile phase. Ferulic acid retention time was comparable to standards for both the enzymatic and control reactions.

Example 5

Ability of Pectinase 162L to hydrolyse chlorogenic acid to caffeic acid in an extract of sunflower meal

Method

Sunflower meal extract was prepared by incubating sunflower meal (10g) with water (100ml) at 45°C and pH8.0 for 20 min. To 20ml of this extract (filtered and adjusted to pH7.0 using hydrochloric acid (1.0M)) was added Pectinase 162L (400 μl; Biocatalysts Ltd.) in a water jacketed reaction vessel stirred via a magnetic follower. A pH Stat was used to hold the reaction at pH 7.0 by dosing aqueous sodium hydroxide (1.0M). The hydrolysis of chlorogenic acid to caffeic acid was monitored by HPLC analysis at t=0, 0.5, 1 and 2 hr.

Time (hr)	Chlorogenic acid (mg/ml)	Caffeic acid (mg/ml)
0	1.49	0.02
0.5	1.1	0.18
1	1.02	0.2
2	0.81	0.23

Example 6

Ability of Biofeed Plus CT to hydrolyse chlorogenic acid to caffeic acid in an extract of sunflower meal

Method

Sunflower meal extract was prepared by incubating sunflower meal (10g)

with water (100ml) at 45°C and pH8.0 for 20 min. To 5ml of this extract (filtered and adjusted to pH7.0 using hydrochloric acid (1.0M)) was added Biofeed Plus CT (10mg; Novo Nordisk). The solution was incubated at 45°C and shaken at 200 rpm. The hydrolysis of chlorogenic acid to caffeic acid was monitored by HPLC analysis at t=0, 1 and 4 hr.

Time (hr)	Chlorogenic acid (mg/ml)	Caffeic acid (mg/ml)
0	1.29	0.01
1	0.68	0.55
4	0.19	0.66

Example 7

Hydrolysis of chlorogenic acid in sunflower meal to caffeic acid by Viscozyme

Method

Sunflower meal (10g) was incubated with water (100ml) at 45°C and pH8.0 for 60 min. The pH was monitored and adjusted to pH8.0 during this period by addition of sodium hydroxide solution (1.0 M). The solution (20ml) containing sunflower meal solids was decanted into a clean flask and Viscozyme (1.4 ml; Novo Nordisk) added. A control reaction without the addition of Viscozyme was also performed. The reactions were incubated at 37°C and shaken at 200 rpm. The hydrolysis of chlorogenic acid to caffeic acid was monitored by HPLC analysis t=3 hr.

Control Reaction		Viscozyme Reaction	
Chlorogenic acid (mg/ml)	Caffeic acid (mg/ml)	Chlorogenic acid (mg/ml)	Caffeic acid (mg/ml)
1.04	0	0	0.65

Example 8

Ability of Celluzyme to release sinapic acid from rape meal

Method

Rape meal (4g) was added to water (40 ml, pH7.0) containing Celluzyme (80 mg; Novo Nordisk) in a water jacketed reaction cell (45°C) and stirred using a magnetic follower. A pH stat was used to hold the reaction at pH7.0 by dosing aqueous sodium hydroxide (1.0 M). After 28.25 hr the amount of sinapic acid extracted was measured by HPLC.

Yield of sinapic acid = 0.757% w/w rape meal.

Yield of sinapic acid = 69% of total sodium hydroxide extractable sinapic acid.

A control reaction (4 g rape meal in 40 ml water) without addition of Celluzyme was performed. After 28.25 hr the amount of sinapic acid extracted was measured by HPLC.

Yield of sinapic acid = 0.184% w/w rape meal.

Yield of sinapic acid = 17% of total sodium hydroxide extractable sinapic acid.

The total sinapic acid content of rape meal was determined by incubating

rape meal (500 mg) in aqueous sodium hydroxide (1.0 M, 10 ml) overnight at 30°C and 200 rpm. The reaction mixture was neutralised with aqueous hydrochloric acid (1.0 M) and the amount of sinapic acid measured by HPLC.

Yield = 1.1% w/w rape meal.

HPLC conditions: C_{18} reverse phase column, 2 ml/min flow rate, 290 nm detector absorbance, acetonitrile/water (20:80) + 1% acetic acid mobile phase. Sinapic acid retention time was comparable to standards for both the enzymatic and control reactions.

Example 9

Nitrogen analysis of sunflower meal: untreated and Celluzyme + extraction treated

Method

Sample 1: Untreated sunflower meal was ground into a fine powder using a mortar and pestle (sample mass 607mg).

Sample 2: Sunflower meal treated with Celluzyme at pH5.0.
Treated meal (2.72g) was repeatedly extracted with ethyl acetate (1 x 2.8ml + 6 x 2.0ml). The extracted meal was dried for 1.5 hr at 50°C, ground to a powder as described for Sample 1 and dried overnight at 50°C (sample mass 441mg).

Sample 3: Sunflower meal treated with Celluzyme at pH7.0 (protein extract removed). Sunflower meal treated with Celluzyme and extracted with ethyl acetate in a similar manner as described in Example 1

(proteinaceous layer formed on extraction was not added back to the meal). The extracted meal was dried for 0.5 hr at 50°C, ground to a powder as described for Sample 1 and further dried for 2 hr at 50°C (sample mass 713mg).

Sample 4: Sunflower meal treated with Celluzyme at pH7.0 (protein extract not removed).

Sunflower meal treated with Celluzyme as described in Example 1 was extracted with ethyl acetate (6 extractions, total vol. 21.9ml; proteinaceous layer formed on extraction was added back to the meal). The extracted meal was dried for 0.5 hr at 100°C, ground to a powder as described for Sample 1 and further dried for 2 hr at 50°C (sample mass 171mg).

Nitrogen analysis:

Samples 1-4 were analysed for Nitrogen using a Fisons NA2000 analyser (combustion at 1800°C followed by quantification on for nitrogen gas by gas chromatographic analysis).

Sample No.		Nitrogen (%)	Protein (%)	Mean Protein (%)
1	Untreated	4.54 4.53	28.39 28.32	28.36
2	Treated pH5.0 (plus protein)	4.15 4.15	25.92 25.92	25.92
3	Treated pH7.0 (minus protein)	4.02 4.04	25.12 25.27	25.2
4	Treated pH7.0 (plus protein)	4.53 4.51	28.34 28.22	28.28

Results demonstrate that removal of the proteinaceous layer formed on ethyl acetate extraction of the meal reduces the estimated protein content of the meal by 3% (samples 3 and 4). If the protein layer is retained with the extracted meal the estimated protein content remains the same as untreated meal (samples 1 and 4). Extraction of the meal at pH5.0 leads to a reduction in protein content compare to untreated and pH7 extracted meal samples.

Example 10

Enzyme selection protocol

Enzyme preparations were tested by adding the enzyme to a 2mg/ml solution of chlorogenic acid in deionised water, the reaction mixture being stirred and heated to 45°C at both pH 7.0 and 5.0 for separate reactions. The reaction was followed on a pH-stat by back titration with 0.01M sodium hydroxide solution and the rate and extent of uptake of base was observed.

Enzymes showing significant hydrolysis of the substrate were then assayed for the production of caffeic acid by HPLC to confirm the desired activity. Preparations which displayed the required hydrolysis were tested further.

Enzyme preparations from the first conversion, above, were then tested for substantial protease activity as follows. A standard 2mg/ml solution of bovine serum albumin in pH 7.5, 0.5M phosphate buffer was treated with enzyme preparation and the resulting mixture stirred at 45°C for 3 hours. The solution was assayed for soluble protein using standard methods such as the Bradford Assay [method of M.M. Bradford, Anal. Biochem, 1974, 72, 248-254] or a commercial protein assay kit such as

that provided by Biorad Laboratories. No substantial protease activity of a test enzyme is indicated when there is no greater loss of measured protein than control reactions which do not contain the test enzyme.

Enzymes which displayed ester hydrolytic activity against chlorogenic acid but no significant hydrolytic activity against bovine serum albumin were regarded as positive in this screen and further tested for hydrolysis of chlorogenic acid in plant meal and non hydrolysis of protein in plant meal. Enzymes displaying these activities are preferred enzymes for use in the methods of the invention.

Example 11

Alternative methods for the removal of the caffeic acid from the sunflower meal reaction mixture

Method:

Sunflower meal was treated with Celluzyme as described in Example 1 but instead of acidification with concentrated hydrochloric acid and extraction using ethyl acetate the following procedures were employed:

i) Separation of the aqueous phase from the meal followed by ethyl acetate extraction. A sample of sunflower meal (approx. 412 g) treated with Celluzyme to hydrolyse chlorogenic acid to caffeic acid was pressed using a Walker Desmond Vigo wine press to separate the aqueous phase (980 ml) from the damp sunflower meal (512 g). The aqueous phase was then acidified to pH3 with concentrated hydrochloric acid and extracted with ethyl acetate (1000 ml + 500 ml), the phases separated, pooled, and the organic phase dried over sodium sulphate and dried with

heating *in vacuo*.

Yield of extract = 1.95 g (caffeic acid content 54% w/w).

Yield of treated sunflower meal (over dried) = 283 g

Optionally the precipitate formed upon acidification of the aqueous phase was removed prior to extraction (see next example).

ii) Separation of the aqueous phase from the meal followed by evaporation.

A sample of Celluzyme treated sunflower meal was pressed as described in (i). The aqueous phase was then dried with heating *in vacuo*.

Yield of extract = 115 g (caffeic acid contact 0.93% w/w).

Yield of treated sunflower meal (over dried) = 288 g.

Example 12

Isolation of protein from sunflower meal treated with Celluzyme

Method:

Sunflower meal was treated as described in Example 1 (total mass 412 g) using Celluzyme but instead of terminating the reaction by addition of hydrochloric acid the following procedure was adopted. The reaction mixture was pressed using a Walker Desmond Vigo wine press to separate the solid meal from the aqueous phase containing soluble protein and caffeic acid. The aqueous phase was adjusted to pH5.0 and then pH3.0 using concentrated hydrochloric acid. The precipitate formed on each pH adjustment was collected by centrifugation and air dried. The protein content of the dried precipitate was determined by the difference between the protein remaining in the aqueous phase before and after pH adjustment. The protein concentration was determined using the Biorad

protein assay and comparing to a standard curve to protein concentrations. The caffeic acid can be recovered from the aqueous phase by organic extraction as described in the previous example.

	Mass of dried precipitate (g)	Protein concentration (% w/w)
pH5.0	19.01	91
pH3.0	15.44	5

Example 13

Extraction of caffeic acid from sunflower meal treated with Celluzyme

Method:

Sunflower meal (825 g) was treated with Celluzyme as described in Example 1 but *n*-butan-1-ol (2500 ml + 1000 ml) was substituted in place of the ethyl acetate. The *n*-butan-1-ol was separated from the aqueous phase, dried over sodium sulphate and dried with heating *in vacuo*.

Yield of extract = 26.29 g (caffeic acid content 16.6% w/w).

Yield of treated sunflower meal (over dried) = 541 g.

Yield of treated sunflower mean + aqueous phase (over dried) = 656 g

Example 14

Enzymatic production of ferulic acid from wheat bran and destarched wheat bran

Method:

Wheat bran (500 g) was added to water (10 ml, pH7.0) containing Celluzyme (10 mg; Novo Nordisk) in a water jacketed reaction cell (45°C) and stirred using a magnetic follower. A pH stat was used to hold the reaction at pH7.0 by dosing aqueous sodium hydroxide (0.1 M). After 4 hr the amount of ferulic acid extracted was measured by HPLC.

Yield of ferulic acid = 0.05% w/w wheat bran.

Yield of ferulic acid = 7% of total sodium hydroxide extractable ferulic acid.

An identical reaction using destarched wheat bran in place of wheat bran was performed. After 4 hr the amount of ferulic acid extracted was again measured by HPLC. Wheat bran was destarched as described by K.G. Johnson *et al.*, Enzyme Microb. Technol. 1988, 10, 403-409. The destarching methods disclosed are generally applicable to all plant materials and can be used to prepare partially or preferably wholly destarched plant material according to a preferred embodiment of the invention.

Yield of ferulic acid = 0.5% w/w wheat bran.

Yield of ferulic acid = 58% of total sodium hydroxide extractable ferulic acid.

The total ferulic acid content of the wheat bran and destarched wheat bran were determined as described in example 8 for sinapic acid in rape meal.

Yield = 0.71% w/w wheat bran.

Yield = 0.86% w/w destarched wheat bran.

HPLC conditions: C_{18} reverse phase column, 2 ml/min flow rate, 290 nm detector absorbance, acetonitrile/water (20:80) + 1% acetic acid mobile phase.

Ferulic acid retention time was comparable to standards for both the enzymatic and control reactions.

Example 15

Enzymatic production of ferulic acid from wheat bran

Method:

Wheat bran (500 mg) was added to phosphate buffer (10 ml, 100mM., pH7.0) in a universal bottle. Celluzyme (4 mg; Novo Nordisk) and Pulpzyme HC® (100 µl; Novo Nordisk) were added and the reaction incubated at 45°C and 200 rpm. After 1 hr the amount of ferulic acid extracted was measured by HPLC.

Yield = 0.26% w/w wheat bran.

Yield = 37% of total sodium hydroxide extractable ferulic acid.

Pulpzyme HC® is a xylanase preparation produced by submerged fermentation of a *Bacillus* strain. It catalyses the hydrolysis of deacylated xylan substrates. It contains endo-1,4-beta-D-xylanase activity (E.C. 3.2.1.8) and is virtually free of cellulase activity.

The commercial product is available from Novo Nordisk at an activity of

500 EXU/g with one xylanase unit (EXU) being defined as the amount of enzyme which, in standard conditions (pH 9.0, 50°C (122°F), 30 minutes incubation), releases a defined amount of dye from dyed RBB xylan. (Details of the analysis method (AF 293.9/1) are available from Novo Nordisk on request.)

Pulpzyme HC® is sold for use in the bleach boosting of kraft pulp. It is classified as a xylanase, CAS No. 9025-57-4, and the xylanase is classified in IUB (International Union of Biochemistry) with EC No. 3.2.1.8.

An identical control reaction without addition of Pulpzyme HC was performed. After 1 hr the amount of ferulic acid extracted was again measured by HPLC.

Yield = 0.04% w/w wheat bran.

Yield = 5% of total sodium hydroxide extractable ferulic acid.

The total ferulic acid content of the wheat bran was determined as described in example 8 for sinapic acid in rape meal.

Yield = 0.71% w/w wheat bran.

HPLC conditions: C_{18} reverse phase column, 2 ml/min flow rate, 290 nm detector absorbance, acetonitrile/water (20:80) + 1% acetic acid mobile phase. Ferulic acid retention time was comparable to standards for both the enzymatic and control reactions.

CLAIMS

1. A method of treating plant material to improve its nutritional value comprising exposing it to a hydrolytic enzyme in an aqueous environment and removing one or more phenolic compounds produced, by solvent extraction.

2. A method of preparing a phenolic compound comprising treating a plant material by exposing it to a hydrolytic enzyme in an aqueous environment and removing the phenolic compound.

3. A method as claimed in Claim 1 or 2 wherein the phenolic compound is selected from caffeic acid, quinic acid, sinapic acid and coumaric acid.

4. A method as claimed in any one of Claims 1 to 3, wherein the enzyme does not exhibit a substantial proteolytic activity.

5. A method as claimed in Claim 4, wherein the enzyme is isolated from *Humicola, Bacillus* and *Aspergillus* species, or is isolated from liver.

6. A method as claimed in Claim 5, wherein the enzyme is selected from one or a combination of two or more enzymes from Pectinase 162L, Biofeed plus CT, Viscozyme, Celluzyme and Pulpzyme HC®, or one or more enzymes having substantially the same enzymatic activity as said enzymes.

7. A method as claimed in Claim 6, wherein the enzyme is Celluzyme.

8. A method as claimed in any preceding claim wherein the enzyme hydrolyses chlorogenic acid to caffeic acid and quinic acid.

9. A method as claimed in any one of Claims 1 to 8 wherein protein in the treated plant material is precipitated and removed.

10. A food product comprising treated plant material or protein obtainable by the method of any one of Claims 1 to 9.

11. Use of plant material or protein as claimed in Claim 10 in the manufacture of a food product for administration to a human or animal.

12. A method as claimed in any one of Claims 1 to 9 further comprising the step of feeding the treated plant material or protein to a human or animal.

13. A method of converting sinapine to sinapic acid and choline comprising hydrolysing the sinapine enzymatically.

14. A method of making sinapic acid and choline as claimed in Claim 13 comprising treating plant material containing sinapine with a hydrolytic enzyme in an aqueous environment and removing the products of hydrolysis, sinapic acid and choline.

15. A method of producing ferulic acid comprising treating plant material with a hydrolytic enzyme in an aqueous environment and removing the ferulic acid product of hydrolysis.

16. A method as claimed in any one of the preceding claims, wherein the plant material is from one or more of sunflower, rice, coffee, rape,

grape, sugar beet and cereal grains such as wheat and corn.

17. A method as claimed in Claim 16, wherein the plant material is cereal grain.

18. A method as claimed in Claim 17, wherein the cereal grain is wheat.

19. A method as claimed in Claim 18, wherein the plant material is wheat germ.

20. A method as claimed in Claim 18 wherein the plant material is wheat bran.

21. A method of making caffeic acid and quinic acid comprising treating plant material containing chlorogenic acid with a hydrolytic enzyme in an aqueous environment and removing the caffeic acid and quinic acid hydrolysis products.

22. A method as claimed in Claim 21, wherein the plant material is from sunflower.

23. A method as claimed in any one of Claims 2, 13, 15 and 21, or a claim dependent on said claims, wherein the hydrolysis products are removed by solvent extraction.

24. A method as claimed in any one of Claims 1 to 9 and 12 to 23 wherein the weight/volume ratio (w/v) of plant material to water is 1:10 or less dilute.

25. A method as claimed in Claim 24, wherein the ratio is 1:5 or less dilute.

26. A method as claimed in Claim 25, wherein the ratio is 1:2.

27. A method as claimed in any one of the preceding claims wherein the plant material is wholly, or at least partially destarched.

28. A method of screening a test enzyme for utility in a method as claimed in Claim 1 or 2 comprising incubating the test enzyme in an aqueous solution of a phenolic compound and assaying the solution for the production of one or more phenolic products of hydrolysis of the phenolic compound, the presence of said products being indicative of suitable hydrolytic activity.

29. A method of screening a test enzyme as claimed in Claim 28 further comprising the step of screening for substantial proteolytic activity by incubating the hydrolytic test enzyme identified in a protein solution and assaying for the presence of protein, substantial proteolytic activity being indicated when there is a greater loss of protein compared to a control reaction which does not contain the test enzyme.

30. A method as claimed in Claim 28 or 29 wherein the phenolic compound is chlorogenic acid and the hydrolysis products are caffeic acid and quinic acid.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01345

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23J1/14 A23L1/211 A23L1/015 A23J3/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A23J A23L C12P A23F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. AGRIC. FOOD CHEM., vol. 32, 1984, pages 123-127, XP002014929 DABROWSKI, SOSULSKI: "QUANTITATION OF FREE AND HYDROLYZABLE PHENOLIC ACIDS IN SEEDS BY CAPILLARY GAS-LIQUID CHROMATOGRAPHY" see page 125, left-hand column; figure 1 see page 126, right-hand column - page 127, left-hand column & J. AGRIC. FOOD CHEM., vol. 26, no. 4, 1978, pages 830-835, SALOMONSSON, THEANDER, AMAN: "QUANTITATIVE DETERMINATION BY GLC OF PHENOLIC ACIDS AS ETHYL DERIVATIVES IN CEREAL STRAWS" see page 833 --- -/--	1-3, 8-14,16, 17,21, 23-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 October 1996	18. 10. 96

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Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/GB 96/01345

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	FOOD CHEMISTRY, vol. 53, no. 4, 1995, pages 427-434, XP000603506 DOMINGUEZ ET AL: "AQUEOUS PROCESSING OF SUNFLOWER KERNELS WITH ENZYMATIC TECHNOLOGY"	1,2,4, 9-12,16, 23
A	see page 429; figure 3	8,21,22
X	FOOD SCIENCE AND TECHNOLOGY, vol. 27, no. 1, 1994, pages 59-66, XP002014930 MICARD ET AL: "studies on enzymic release of ferulic acid from sugar-beet pulp"	1,2,4,5, 15,16, 20,23,28
A	see page 61; table 1	6
X	ENZYME AND MICROBIAL TECHNOLOGY, vol. 14, no. 11, 1992, pages 875-884, XP000603441 CASTANARES ET AL: "PURIFICATION AND PROPERTIES OF A FERUOYL/P-COUMAROYL ESTERASE FROM THE FUNGUS PEN. PINOPHILUM" see page 878, right-hand column	1-5,15, 28
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A	see page 1082	23
X	US,A,4 426 448 (KIKKOMAN CORPORATION) 17 January 1984 see column 1-2; claims 1,2 see column 6-7	1-5,8, 10-12, 16,21, 23-25, 28-30

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International Application No
PCT/GB 96/01345

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EP,A,0 503 650 (TSUNO FOOD INDUSTRIAL CO LTD) 16 September 1992 see column 1-3; claims 1-3,5,9-11,13,14,17,21; example 1	1,2, 15-17,23

A	US,A,4 158 656 (CANADIAN PATENTS AND DEVELOPMENT LTD) 19 June 1979 see column 2-4; claims 1,5,6,9,10; figures 1,2	1,3, 9-13,16, 23-25

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International Application No
[redacted]/GB 96/01345

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